Plasma-SeqSensei™

Sample Preparation Guide

Manual guideline

Version 2

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en

Glossary of Symbols			
	Manufacturer		Caution
	Consult instructions for use		Keep dry
	Temperature limit		Humidity limit

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1 Introduction

Tumour cells undergoing apoptosis, necrosis, or metabolic secretion release small amounts of their DNA into the blood stream. The tumour-specific fraction of circulating cell-free DNA (cfDNA) is called circulating tumour DNA (ctDNA) and contains the genetic information for the primary tumour and even metastases. A multitude of research studies and trials have demonstrated the clinical application of ctDNA profiling at different stages of cancer treatment, including therapy selection, prognosis, and monitoring (1).

Various NGS-based technologies are available for ctDNA detection. However, due to sequencing and PCR bias and errors, most of them are inappropriate for the detection of rare variants. Plasma-SeqSensei™ is a novel NGS-based technology that implements unique molecular identifiers (UID) in the sequencing workflow. This results in a significant error reduction, leading to an ultra-high sensitivity of the Plasma-SeqSensei™ technology (2).

To achieve high sensitivity and best results, the preparation of cfDNA from blood samples has to be performed according to the tested procedure described in ► chapter *4 Procedure,* page 12/30.

1.1 Intended purpose

The purpose of this sample preparation guide is to assist users of the Plasma-SeqSensei™ *Assay-Specific* Kits with the preparation of cfDNA from blood samples. This guide contains the procedures for plasma preparation, cfDNA purification, as well as cfDNA quantification with Qubit™. The workflow starts with blood samples collected in Streck Cell-Free DNA BCT® CE for the use in liquid biopsy applications such as Plasma-SeqSensei™ *Assay-Specific* Kits and Next-Generation Sequencing (NGS) technologies.

1.2 Workflow

The Plasma-SeqSensei™ workflow is sectioned into multiple steps that must be adhered to for optimal results. Figure 1 describes this process and includes steps for the sample preparation, as well as guidance for specific Instructions for Use (IFUs) to be followed from blood collection to sequencing results.

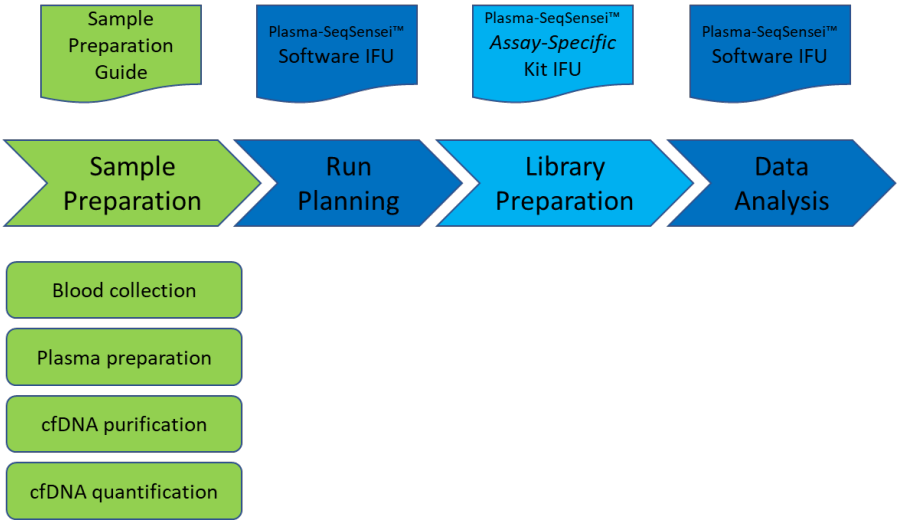

Figure 1: Plasma-SeqSensei™ process, including workflow steps described in Sample Preparation Guide and other required documents.

2 Reagents, consumables and equipment

The workflow described in this guide requires only non-supplied material to prepare blood samples for the use of the Plasma-SeqSensei™ *Assay-Specific* Kits. For the description and use of the Plasma-SeqSensei™ *Assay-Specific* Kit components, please refer to the Plasma-SeqSensei™ *Assay-Specific* Kit IFU (www.mysysmex.com).

2.1 Non-supplied material

Products, where details about manufacturer/vendor and order number are provided in Table 1, Table 2, and Table 3, are essential for the assay and must not be interchanged by products with comparable quality and/or properties.

Table 1: Essential material not provided for Plasma-SeqSensei™ sample preparation

Material	Product
Reagents and kits	* QIAamp® Circulating Nucleic Acid Kit, QIAGEN, #55114
	Ethanol (EtOH) ≥ 99.8 %, p.a.
	2-Propanol ≥ 99.8 %, p.a.
	1X Phosphate buffered saline solution (PBS), (pH 7.4 ± 0.2, without Ca^{2+} and Mg^{2+})
	* Qubit™ 1X dsDNA HS Assay Kit, Thermo Fisher, #Q33230 (100 rxns) or #Q33231 (500 rxns)

* Essential components; must not be interchanged by products with comparable quality and/or properties.

2.2 Consumables

Table 2: Consumables required for Plasma-SeqSensei™ sample preparation

Laboratory equipment	Product
Blood collection device	* Streck Cell-Free DNA BCT® CE, 10.0 ml volume, Streck #218996, #218997 or #230244 or equivalent
Pipette tips / serological pipettes	Aerosol-resistant sterile pipette tips with filters 10, 200, 1000 µl
	Serological pipette, 5, 10, 25 ml, sterile
Reaction tubes	50, 15, 2, 1.5 ml tubes
	* LoBind® DNA tubes 1.5 ml, Eppendorf, #0030108051
	* Qubit™ Assay Tubes, Thermo Fisher, #Q32856
	Cryogenic vial (polypropylene, 3-5 ml recommended)
Safety equipment	Protective glasses
	Protective coats, sleeves, disposable shoe covers, gloves
Miscellaneous	* 3 ml Extension tubes for QIAvac vacuum manifolds, QIAGEN, #19587
	* VacConnectors (500) for QIAvac vacuum manifolds, QIAGEN, #19407

* Essential components; must not be interchanged by products with comparable quality and/or properties.

2.3 Equipment

Table 3: Equipment required for Plasma-SeqSensei™ sample preparation

Laboratory equipment	Product
Electronic instruments	Centrifuge for 1.5/2 ml tubes, capable of 20,000 × g, fixed angle rotor
	Centrifuge for plasma preparation, capable of 6,000 × g, swing bucket rotor (fixed angle rotor for second spin optional), with low deceleration/acceleration ramp, with adaptors for 15 ml tubes
	Adaptors for fixed-angle rotors for cryogenic vials
	Minicentrifuge, capable of ≤ 2,000 × g

2 Reagents, consumables and equipment

Laboratory equipment	Product
	Vortexer with inserts for tubes
	Thermometer
	Waterbath
	Heat block with inserts for 2 ml tubes
	Freezer, -15 °C to -30 °C
	Refrigerator, 2 °C to 8 °C
	Deep freezer, -70 °C to -100 °C
	DNA workstation/PCR cabinet
	Fume hood (strongly recommended)
	Class II Biological Safety Cabinets (strongly recommended)
	* QIAGEN Connecting System
	* QIAvac 24 Plus System
	Vacuum pump (230 V, 50 Hz)
	* Qubit™ 3 or 4 Fluorometer
Pipettes	Pipette 1000 µl, 200 µl, 10 µl
	Pipettor 5 to 100 ml
	Disposable bulb pipettes
Racks	50 ml, 15 ml, 1.5/2 ml tube rack
	Freezer storage boxes
Miscellaneous	Ice bucket with ice or cooling beads or cooling rack for 15/50 ml tubes
	Stopwatch

* Essential components; must not be interchanged by products with comparable quality and/or properties.

3 Storage and handling

3.1 Shipping conditions

The preparation of the blood sample for the use of the Plasma-SeqSensei™ *Assay-Specific* Kits requires third-party products that are not supplied. For optimal shipping conditions, follow the manufacturer's instructions for non-supplied material.

Important: *Contrary to the manufacturer's instructions, samples collected in Streck Cell-Free DNA BCT® CE are to be stored for no longer than 6 days.*

3.2 General handling precautions

Ensure that temperature and humidity within the laboratories remain between 15 °C and 25 °C and between 20 % and 85 %, respectively (reduce risk of condensation/evaporation).

Do not eat, drink, or smoke in laboratory areas. Perform equipment maintenance according to manufacturer's instructions.

Decontaminate and dispose of all reagents, specimens, and associated supplies in accordance with government regulations applicable in your location. For accurate and reproducible results, it is essential to avoid contamination with foreign DNA, especially PCR products from previously run samples. The amplified products from previous experiments constitute the most common source of DNA contamination.

3.3 Warnings and precautions

Avoid contact of reagents with the skin, eyes, or mucous membranes. For additional warnings, precautions, and specific measures, refer to the manufacturer's safety data sheets (SDS).

3.3.1 Specific measures

First aid measures:

- ***General advice*:** In case of persisting effects, consult a physician. Remove contaminated clothing and shoes immediately, and launder thoroughly before reusing.
- ***If inhaled:*** Remove the affected person from the immediate area. Ensure supply of fresh air.
- ***In case of skin contact:*** Wash off the affected area with soap and plenty of water.
- ***In case of eye contact***: Remove contact lenses. Rinse the eye thoroughly under running water keeping eyelids wide open for at least 10 to 15 minutes. Protect the unaffected eye.
- ***If swallowed*:** Call a doctor immediately. Do not induce vomiting. Never give anything by mouth to an unconscious person.

3.3.2 Handling and storage

General protective and hygiene measures

Do not eat, drink, or smoke in the laboratory and ensure that good hand washing technique is employed before leaving. Do not inhale vapours. Avoid contact with eyes and skin. Remove soiled or soaked clothing immediately.

Precautions for safe handling

Product handling risks must be minimised by taking the appropriate protective measures and preventative actions. The working process should be designed to rule out the release of the hazardous substances or skin contact as far as it is possible.

Advice on protection against fire and explosion

No special measures necessary.

Conditions for safe storage, including any incompatibilities

Keep the container tightly closed in a dry and well-ventilated place. Opened containers must be carefully resealed and kept upright to prevent leakage.

3.3.3 Reagent handling precautions

To ensure proper use and disposal of reagents and to avoid contamination of reagents, follow the precautions listed below:

- Do not use expired or incorrectly stored reagents.
- Prepare reagents according to manufacturer's instructions.
- Reagents should be used only with other reagents from the same kit.
- Reagents from different kits or lots must never be pooled or interchanged.
- Record the open date and mark tubes after each use to ensure reagents are not expired or used beyond the recommended number of freeze-thaw cycles.
- Avoid contamination of reagents by changing gloves frequently. Always change gloves between the handling of reagents and specimens.
- Dispose of unused reagents and waste according to country, federal, state, and local environmental regulations.

3.3.4 Safety and contamination precautions

Follow the precautions listed below to maintain a laboratory environment free of DNA contamination and to ensure safety of all personnel:

- Separate the workspaces used for pre-PCR and post-PCR and adhere to a unidirectional workflow from 'clean' (pre-amplification) to 'dirty' (post-amplification) areas.
- Ensure that dedicated equipment (including pipettes), supplies, reagents, biohazard waste containers, and lab manuals are present in each working area. Never exchange these materials between pre- and post-PCR work areas. We recommend colour-coding or labelling of equipment, supplies, and reagents to identify those that belong to a particular area.
- Wear appropriate personal protective equipment throughout the procedure.
    - Wear a lab coat (preferably disposable) and disposable powder-free gloves at all times when working in the pre-PCR and post-PCR areas.

  - To prevent contamination, change gloves frequently between handling specimens and reagents and after contact of the outside of the gloves with the skin.
  - Wear protective eyeglasses at least during plasma preparation and DNA extraction.
  - Wear disposable shoe covers or change shoes between pre- and post-PCR laboratories and wear disposable arm protection sleeves (required in the pre-PCR laboratory).
- When exiting pre-PCR and post-PCR laboratory areas, remove and discard personal protective equipment.
- Handle all specimens as potentially infectious material. If a spill occurs, it is recommended to clean the affected area first with detergent/disinfectant and water and then with ~0.5 % sodium hypochlorite (bleach) solution prepared using deionized water.

  Note: *Commercial liquid household bleach (e.g. Clorox brand) typically contains sodium hypochlorite at a concentration of 5.25 %. A 1:10 dilution of household bleach will produce a 0.5 % sodium hypochlorite solution.*

- Use dedicated PCR cabinets for pipetting steps.
- After use, clean PCR cabinets with quaternary ammonium compounds disinfectant (such as RHEOSEPT-WD plus or equivalent) followed by a product designed for removing nucleic acids and nucleases (such as Roti® Nucleic Acid-Free or equivalent).
- After use, clean PCR workspaces with a product designed for removing nucleic acids and nucleases (such as Roti® Nucleic Acid-Free or equivalent).
- Decontaminate the safety cabinet, PCR workspaces, and labware (pipettes, tube racks, or other equipment) with ultraviolet (UV) light after use. To ensure UV radiation is effective, regularly clean UV bulbs from the accumulating residue.
- Use only aerosol-resistant sterile pipette tips with filters (lot certified, RNase-, DNase-, and pyrogen-free).
- Use only PCR-grade reagents and tubes.
- Keep only one specimen tube or reagent tube open at a time.
- To prevent contamination of multiple-use reagent solutions, prepare working aliquots according to instructions and avoid direct pipetting.

4 Procedure

4.1 Blood collection and processing

The Plasma-SeqSensei™ *Assay-Specific* Kits have been developed using Streck Cell-Free DNA BCT® CE blood collection tubes. Collect and handle blood according to the manufacturer's instructions. Streck Cell-Free DNA BCT® may be used for samples that are shipped for testing or for in-house laboratory testing.

Note: *Do not under- or overfill your Streck blood collection tubes as this can influence the product performance and analytical results.*

Note: *Avoid temperatures at or below 6 °C and at or above 37 °C during shipment, as a soft cell layer may form (≤ 6 °C > 12 h duration) or haemolysis (≥ 37 °C > 12 h duration) can occur.*

Recommendation: *Store blood samples in Streck Cell-Free DNA BCT® CE blood collection tubes at room temperatures (15 °C to 25 °C).*

Important: *Start plasma preparation no later than 6 days after the date of the blood draw.*

Note: *Haemolysis of blood cells increases with storage time of the blood and can slowly increase the level of genomic DNA (unspecific background). Prepare plasma as soon as possible to achieve the highest sensitivity possible with your samples.*

4.2 Plasma preparation instructions (whole blood)

Plasma preparation steps must be carried out in quick succession, omitting pauses between individual steps.

Table 4: Required material for plasma preparation from blood

Material	Product
Blood collection device	* Streck Cell-Free DNA BCT® CE, 10.0 ml volume, Streck #218996, #218997, #230244 or equivalent
Pipettes	Disposable bulb pipettes

Material	Product
Pipette tips	Aerosol-resistant sterile pipette tips with filters 200, 1000 µl
Reaction tubes	15 ml tubes
	Cryogenic vials (polypropylene, 3-5 ml recommended)

Preparation:

- Turn on the biosafety cabinet and ensure its proper functioning.
- Clean the biosafety cabinet and equipment with DNA-removing agent (e.g. DNA away) and disinfecting agent (e.g. 70 % EtOH).
- Label two 15 ml conical tubes per blood sample.
- Label cryotubes (for up to 8 ml) per blood sample if storage in the deep freezer is required.

Plasma preparation procedure:

1. Centrifuge the blood tube at temperatures between 15 °C and 25 °C for 10 min at 1,600 x g using a swing-out rotor.

   Note: *To prevent disruption of the cell layer, use a low deceleration ramp of the centrifuge.*

2. After centrifugation, remove tubes carefully from the centrifuge (avoid turbulences).

3. Visually check plasma fraction (supernatant).

   Note: *Soft cell layer may be observed for samples stored at or below 6 °C for a prolonged amount of time (> 12 hours). Haemolysis (light pink or reddish plasma) may be observed for samples stored at or above 37 °C for a prolonged amount of time (>12 hours).*

   Important: *We do not recommend analysis of haemolytic samples or samples without a clear plasma fraction due to increased risk of elevated genomic DNA background.*

4. Without disturbing the cellular layer, transfer plasma (supernatant) to a fresh 15 ml conical tube by pipetting along the tube wall using a single channel pipette or a disposable bulb pipette (try to avoid strong turbulences by pipetting slowly). Leave at least 500 µl of residual plasma over the cell layer to avoid its disruption.

Note: *If soft cell layer is present, transfer only the clear plasma fraction and leave at least 500 µl of residual plasma over the cell layer. If the sample does not show a clear plasma fraction, samples should not be used.*

5. Centrifuge the plasma in the 15 ml centrifuge tube at 15 °C to 25 °C for 10 min at 6,000 x g using a swing-out or fixed angle rotor to separate any residual blood cells from the plasma.

   Note: *Use a low deceleration ramp of the centrifuge.*

6. After centrifugation, remove tubes carefully from the centrifuge (avoid turbulences).

7. Without disturbing the cell pellet, transfer plasma (supernatant) to a fresh 15 ml centrifuge tube by pipetting along the tube wall using a single channel pipette or disposable bulb pipette (try to avoid strong turbulences by pipetting slowly). Leave a residual volume of about 300 µl (~7 mm) on the bottom of the tube to avoid contaminating the plasma with cells.

8. Gently mix plasma by pipetting up and down 5 times using a single channel pipette set to 1 ml volume or a disposable bulb pipette.

9. If not directly proceeding with 'Purification of circulating DNA from plasma' steps, transfer plasma into pre-labelled cryogenic vials and immediately place cryogenic vials in an upright position in a fridge at 2 °C to 8 °C for up to 7 days (short term storage) or in a freezer at a temperature between -70 °C and -100 °C for long-term storage (up to a maximum of 24 months).

   Note: *Frozen plasma samples must be shipped on dry ice.*

4.3 Purification of circulating DNA from plasma

For the Plasma-SeqSensei™ *Assay-Specific* Kit, the purification of circulating cell-free DNA (cfDNA) from plasma must be performed using the QIAamp® Circulating Nucleic Acid Kit #55114 (QIAGEN). Any volume between 2 and 4 ml plasma per sample may be processed.

Important: *Deviating from QIAGEN protocol, do NOT add carrier RNA during the DNA purification workflow and use a 60 min water bath incubation*

at 60 °C (±1 °C) as stated in the Streck Cell-Free DNA BCT® CE IFU. The DNA elution follows a two-step procedure.

Table 5: Required material for cfDNA purification from plasma

Material	Product
Reagents and kits	* QIAamp® Circulating Nucleic Acid Kit, QIAGEN, #55114
	Ethanol (EtOH) ≥ 99.8 %, p.a.
	2-Propanol ≥ 99.8 %, p.a.
	1X Phosphate buffered saline solution (PBS), (pH 7.4 ± 0.2, without Ca^{2+} and Mg^{2+})
Pipette tips/serological pipettes	Aerosol-resistant sterile pipette tips with filters 20, 200, 1000 µl
	Serological pipette, 5, 10, 25 ml, sterile
Reaction tubes	50, 15, 2, 1.5 ml tubes
	* LoBind® DNA tubes 1.5 ml, Eppendorf, #0030108051
Miscellaneous	* 3 ml Extension tubes for QIAvac vacuum manifolds, QIAGEN, #19587
	* VacConnectors (500) for QIAvac vacuum manifolds, QIAGEN, #19407

Preparation:

- Turn on the biosafety cabinet and ensure its proper functioning.
- Clean the biosafety cabinet and equipment with DNA-removing agent (e.g. DNA away) as well as disinfecting agent (e.g. 70 % EtOH).
- Set the water bath temperature to 60 °C (±1 °C) and verify the temperature of the water with an external thermometer.
- Set the heat block temperature to 56 °C (±1 °C) and verify the temperature of the blocks with an external thermometer.
- Check the fluid level in liquid waste bottle of the vacuum system as well as the biohazard waste container. Empty flask or use new container if necessary.
- Before starting the work, turn on the vacuum pump to verify that it reaches the required vacuum pressure (between -800 and -900 mbar). If not, check connections of tubing or change filter.

When using a new kit, prepare the solutions as described below.

Buffer ACB

- Before use, add 200 ml isopropanol (2-Propanol ≥ 99.8 %, p.a.) to 300 ml Buffer ACB concentrate to obtain 500 ml Buffer ACB. Mix well.

Buffer ACW1

- Before use, add 25 ml EtOH (≥ 99.8 %, p.a.) to 19 ml Buffer ACW1 concentrate to obtain 44 ml Buffer ACW1. Mix well.

Buffer ACW2

- Before use, add 30 ml EtOH (≥ 99.8 %, p.a.) to 13 ml Buffer ACW2 concentrate to obtain 43 ml Buffer ACW2. Mix well.

DNA Purification:

The following steps are performed in the sample preparation area in the pre-PCR laboratory.

Note: *In the following steps, ▲ denotes sample volumes of 2.0-3.0 ml plasma and ● denotes sample volumes of 3.1-4.0 ml plasma.*

1. Allow plasma samples to thaw at 15 °C to 25 °C for approximately 15 to 20 min. Do not mix or vortex.

   Note: *The time varies depending on the sample volume and the ambient temperature. Once the samples are thawed, i.e. no more ice is visible in the tube, proceed immediately with the following steps.*

2. During this time, prepare all reagents. Fill a collection tube (from the QIAamp® Circulating Nucleic Acid Kit) with ~1 ml of PBS for each sample (to adjust the volume of plasma aliquots).

   Note: *For each sample aliquot, prepare a separate tube with PBS.*

3. Label one 50 ml tube per sample and pipette ▲ 300 µl or ● 400 µl QIAGEN proteinase K into each 50 ml tubes depending on total plasma volume. Place in the biosafety cabinet.

4. When plasma samples are thawed, evaluate the plasma for haemolysis.

Important: *We do not recommend analysis of haemolytic samples due to increased risk of elevated genomic DNA background.*

5. Spin down cryogenic vials containing plasma (brought to a temperature between 15 °C to 25 °C before) at 1,000 x g using a fixed angle rotor. Stop centrifugation once speed is reached.

   Note: *Ensure that the following steps 176 to 8 are performed in a biosafety cabinet in the pre-PCR area.*

6. If centrifuged plasma supernatant volumes are below ▲ 3.0 ml or below ● 4.0 ml, transfer supernatant to a new labelled tube and adjust volumes with PBS from step 2 to ▲ 3.0 ml or ● 4.0.

7. Add ▲ 3.0 ml or ● 4.0 ml of plasma supernatant per sample to the respective tubes containing proteinase K.

   Note: *When pipetting, avoid disrupting the potential pellet and leave about 30 µl of plasma at the bottom of the tube.* ***When combining multiple aliquots, open the tubes from only one test subject at a time,*** *i.e. the tubes from which the plasma is to be taken and the tube containing the proteinase K to which the plasma is added.*

8. Add ▲ 2.4 ml or ● 3.2 ml Buffer ACL (without carrier RNA) and close the cap.

9. Mix the tubes by pulse vortexing for 30 s.

   Note: *Pulse vortexing means vortexing in short intervals.*

   Note: *Make sure a visible vortex forms in the tube to ensure efficient lysis.*

10. Incubate at 60 °C (±1 °C) in a water bath for 60 min (±2 min).

11. In the meantime, fill the ice bucket with ice or get the cooling rack from the 2 °C to 8 °C fridge or the cooling beads from the -15 °C to -30 °C freezer and label the required tubes:

    a. One QIAamp® Mini column per sample.

    b. 1.5 ml tube per sample.

12. Add ▲ 5.4 ml or ● 7.2 ml Buffer ACB to the lysate in the tube.

13. Close the tube and mix thoroughly by pulse vortexing for 15 s to 30 s.

Note: *The following steps are independent of the initial sample volume. No further differentiation between 2-3 ml and 3.1-4 ml is indicated.*

14. Incubate the samples on ice or cooling beads for 5 min (±1 min). If using a cooling rack from the 2 °C to 8 °C fridge, please incubate for exactly 8 min.
15. Meanwhile, prepare the QIAvac 24 Plus vacuum system:
    - Insert the appropriate number of VacConnectors in the slots.
    - Place the labelled QIAamp® Mini columns into the VacConnectors.
    - Place a column extender (extension adapter) on each QIAamp® Mini column.
16. After incubation on ice, centrifuge the sample tube at 1,000 x g for 30 s to remove the condensate from the lid.
17. With the main vacuum valve still closed, turn on the vacuum pump (set to between -800 and -900 mbar).
18. Carefully transfer the lysate into the column extender (serological pipette). Discard tubes.

    Note: *To prevent cross-contamination, avoid moving the pipette over the column extenders of other QIAamp Mini columns.*
19. Ensure that the vacuum pressure is in the range between -800 and -900 mbar.
20. Open the main vacuum valve and let the lysate pass through the columns completely.
21. Close the main vacuum valve (vacuum remains on) and **maintain vacuum** pressure in QIAvac 24 Plus.
22. Pipette 600 µl Buffer ACW1 into each column extender.

    Note: *To avoid cross-contamination, use a fresh pipette tip for each sample and avoid moving pipette tips over the column extender of other samples.*
23. Open the main vacuum valve and let the buffer flow through the columns completely.

24. Close the main vacuum valve (vacuum remains on) and **release vacuum** from the QIAvac 24 Plus. Remove the column extenders and discard them.

    Note: *To avoid cross-contamination, be careful not to remove the column extenders over other samples/columns.*

25. Apply 750 µl Buffer ACW2 to each QIAamp® Mini column.

    Note: *To avoid cross-contamination, use a fresh pipette tip for each sample and avoid work over the QIAamp® Mini columns containing other samples.*

26. Open the main vacuum valve and let the buffer flow through the columns completely.

27. Close the main vacuum valve (vacuum remains on) and **release vacuum** from the QIAvac 24 Plus.

28. Apply 750 µl EtOH (≥ 99.8 %, p.a.) to each QIAamp® Mini column.

    Note: *To avoid cross-contamination, use a fresh pipette tip for each sample and avoid work over the QIAamp® Mini columns containing other samples.*

29. Open the main vacuum valve and let the EtOH flow through the columns completely.

30. Close the main vacuum valve and switch off the vacuum pump.

31. Close the QIAamp® Mini columns, remove them from the QIAvac 24 Plus and place them into a 2 ml collection tube. Discard VacConnectors.

32. Centrifuge the samples in Eppendorf Centrifuge 5430 or equivalent at full speed (20,000 x g) for 3 min (±0.5 min).

33. Place each QIAamp® Mini column into a new 2 ml collection tube. Discard the used 2 ml tube.

34. Open each lid and incubate the assembly on a heat block at 56 °C (±1 °C) for 10 min (±1 min) to dry the membrane completely.

35. Place each QIAamp® Mini column into a clean labelled 1.5 ml LoBind® tube and discard the 2 ml collection tubes.

36. Carefully apply 70 µl Buffer AVE to the centre of the membrane without touching the membrane.

    Note: *Use a fresh pipette tip for each sample.*

37. Close each lid and incubate at 15 °C to 25 °C for 3 min (±0.5 min).
38. Centrifuge in Eppendorf Centrifuge 5430 or equivalent at full speed (20,000 x g) for 1 min to elute the nucleic acids.
39. Without touching the membrane, carefully apply an additional 70 µl Buffer AVE to the centre of each membrane.

    Note: *Use a fresh pipette tip for each sample.*

40. Close each lid and incubate at 15 °C to 25 °C for 3 min (±0.5 min).
41. Centrifuge in Eppendorf Centrifuge 5430 or equivalent at full speed (20,000 x g) for 1 min to elute the nucleic acids.
42. Discard the QIAamp® Mini columns and close the LoBind® tubes.
43. Store cfDNA in the pre-PCR laboratory at temperatures between 2 °C and 8 °C up to 7 days or between -15 °C and -30 °C for longer storage times. The purified DNA is stable for at least 1 year when stored between -15 °C and -30 °C.

4.4 Sample quantification (Qubit™)

The Qubit™ Assay is used to quantify cfDNA amounts extracted from plasma samples. DNA amounts should be reported in ng/µl.

Note: *Qubit measurement of the samples merely represents a rough approximation of input DNA to determine the sample load. The final and possibly differing quantification of the samples will occur during the sequencing of the library using the internal quantifier (Quantispike).*

The Plasma-SeqSensei™ *Assay-Specific* Kits have been developed with Qubit™ 1X dsDNA HS Assay (assay range: 10 pg/µl – 100 ng/µl).

Table 6: Required material for sample quantification using Qubit™

Material	Products
Reagents and kits	* Qubit™ 1X dsDNA HS Assay Kit, Thermo Fisher, #Q33230 (100 rxns) or #Q33231 (500 rxns)
	* Qubit™ Assay Tubes, Thermo Fisher, #Q32856
Pipette tips	Aerosol-resistant sterile pipette tips with filters 10, 200 µl

The following steps are performed in the sample preparation area in the pre-PCR laboratory.

Note: *If cfDNA has been stored for longer periods of time, evaporation of liquid can occur. Verify that you have sufficient volume left for Qubit quantification and UID PCR set-up. If volume is not sufficient, please use Buffer AVE (QIAGEN) to increase volume.*

Qubit™ Measurement:

Perform protocol as described in the manual provided by the manufacturer using 5 µl of sample (see Table 7).

Table 7: Determination of DNA concentration with Qubit™

	DNA input	Qubit™ 1X dsDNA HS working solution (Component A)
Standard	10 µl	190 µl
Sample	5 µl	195 µl

To calculate the **total DNA input of the library** including all samples (without controls) use the following equation:

$$\boldsymbol{DNA\ input\ per\ sample} = measured\ concentration\ in\ ng/\mu l * 116\ \mu l$$

$$\boldsymbol{Total\ DNA\ input} = \sum DNA\ input\ of\ all\ samples\ (in\ 116\ \mu l)$$

Note: *Each **positive control contains 4.3 ng of DNA**, which needs to be considered when calculating the total DNA input for each sequencing run. The positive control DNA will be automatically included in the calculation by the run planning module of the Plasma-SeqSensei™ Software.*

Purified cfDNA can be stored in the pre-PCR laboratory at temperatures between 2 °C and 8 °C for up to 7 days or between -15 °C and -30 °C for longer storage times. The purified DNA is stable for at least 1 year.

Next Steps:

Refer to the Plasma-SeqSensei™ Software IFU for run planning and the Plasma-SeqSensei™ *Assay-Specific* Kit IFU for the preparation of the sequencing library (see Figure 1).

5 Technical assistance

If problems occur during sample preparation, please refer to the manufacturer's troubleshooting guide if available or contact the manufacturer of the product for assistance.

6 Glossary and terminologies

Term	Definition
BCT	Blood Collection Tubes
cfDNA	Cell-free DNA
ctDNA	Circulating tumour DNA
DNA	Desoxyribonucleic acid
EtOH	Ethanol
IFU	Instructions for use
NGS	Next-generation sequencing
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SDS	Safety data sheet
UID	Unique (molecular) identifier
UV	Ultraviolet

7 References

1) Stewart CM, Kothari PD, Mouliere F, Mair R, Somnay S, Benayed R, Zehir A, Weigelt B, Dawson SJ, Arcila ME, Berger MF, Tsui DW. The value of cell-free DNA for molecular pathology. J Pathol. 2018 Apr;244(5):616-627. doi: 10.1002/path.5048. Epub 2018 Mar 12. PMID: 29380875; PMCID: PMC6656375.

2) Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011 Jun 7;108(23):9530-5. doi: 10.1073/pnas.1105422108. Epub 2011 May 17. PMID: 21586637; PMCID: PMC3111315.

8 Copyrights and trademarks

9 Revision history

Document version	Date	Change description	Section
R2	December 2024	Reduction of upper laboratory temperature limit and specification of room temperature (15 °C to 25 °C)	3.2 4.1 to 4.3
		Update of recommended shipping and storage temperatures of blood samples	4.1
		Restructure Chapter 4.2 and update notes on samples not to use	4.2 and steps 3-4
		Restructure initial steps of Chapter 4.3	4.3
		Reduction of centrifugation speed	4.3 step 16
		Information about handling of evaporation of cfDNA samples	4.4
		Addition of revision history table	9
		Minor corrections, spelling, layout	
R1	April 2022	N/A	

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